Interplay between S-Cyclin-dependent Kinase and Dbf4-dependent Kinase in Controlling DNA Replication through Phosphorylation of Yeast Mcm4 N-Terminal Domain

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Cyclin-dependent (CDK) and Dbf4-dependent (DDK) kinases trigger DNA replication in all eukaryotes, but how these kinases cooperate to regulate DNA synthesis is largely unknown. Here, we show that budding yeast Mcm4 is phosphorylated *in vivo* during S phase in a manner dependent on the presence of five CDK phosphoacceptor residues within the N-terminal domain of Mcm4. Mutation to alanine of these five sites (*mcm4-5A*) abolishes phosphorylation and decreases replication origin firing efficiency at 22°C. Surprisingly, the loss of function *mcm4-5A* mutation confers cold and hydroxyurea sensitivity to DDK gain of function conditions (*mcm5/bob1* mutation or DDK overexpression), implying that phosphorylation of Mcm4 by CDK somehow counteracts negative effects produced by ectopic DDK activation. Deletion of the S phase cyclins Clb5,6 is synthetic lethal with *mcm4-5A* and mimics its effects on DDK up mutants. Furthermore, we find that Clb5 expressed late in the cell cycle can still suppress the lethality of *clb5,6Δ bob1* cells, whereas mitotic cyclins Clb2, 3, or 4 expressed early cannot. We propose that the N-terminal extension of eukaryotic Mcm4 integrates regulatory inputs from S-CDK and DDK, which may play an important role for the proper assembly or stabilization of replisome–progression complexes.

INTRODUCTION

Eukaryotic chromosome replication initiates throughout S phase from multiple origins, and it is controlled in large part by cyclin-dependent kinases (CDKs). CDKs have both positive and negative roles for DNA replication. They promote the initiation of DNA synthesis from competent origins, but they also prevent the reassembly of prereplicative complexes (preRCs) at origins that have already fired. This inhibition of preRC formation by CDKs allows chromosome replication to be coupled to the cell cycle (Diffley, 2004). PreRC assembly is well understood: it begins with origin recognition complex binding to origin DNA, followed by recruitment of Cdc6 and Cdt1, which in turn permit loading of the Mcm2-7 complex, the likely replicative helicase (Blow and Dutta, 2005; Takeda and Dutta, 2005). Binding of Cdc6 and Cdt1 is a highly regulated step ensuring that origin licensing is restricted to the G1 phase and that origins do not fire twice during the same cell cycle (Blow and Dutta, 2005). CDKs inhibit origin licensing by targeting several preRC

components (Cdc6, Cdt1, and Mcm3) for degradation or nuclear exclusion (Liku *et al.*, 2005). Phosphorylation by CDK of Orc2 and Orc6 in *Saccharomyces cerevisiae* (Nguyen *et al.*, 2001) and Orp2 in *Schizosaccharomyces pombe* (Vas *et al.*, 2001) also participates in inhibiting preRC formation during the S-to-M phase period.

Besides preventing preRC assembly CDKs also have a positive, yet less well understood role for origin firing. The maturation of preRCs requires activation of two evolutionary conserved S/T protein kinases: Dbf4-Cdc7 (Dbf4-dependent kinase or DDK) and S-phase CDK (Clb5,6-Cdk1 in *S. cerevisiae* or CycE,A-Cdk2 in higher eukaryotes). These two kinases promote the recruitment of the Sld3-Cdc45 and Sld2-Dpb11 heterodimers, the GINS complex and finally, RPA and DNAPol α /primase to the site of initiation (Tanaka and Nasmyth, 1998; Zou and Stillman, 2000; Kamimura *et al.*, 2001; Masumoto *et al.*, 2002; Takayama *et al.*, 2003). Some of these initiation factors (Mcm2-7, Cdc45, and GINS) move along with replication forks, indicating that they may be part of the active helicase complex (Aparicio *et al.*, 1997; Labib *et al.*, 2000; Tercero *et al.*, 2000; Takayama *et al.*, 2003). Accordingly, minichromosome maintenance (MCM) and Cdc45 are both required for DNA unwinding in *Xenopus* egg extracts (Pacek and Walter, 2004). The GINS complex maintains association of MCM helicase with Cdc45 and other replication factors, such as the checkpoint mediator Mrc1, the fork-pausing complex Tof1-Csm3 as well as DNA polymerase-associated proteins (Gambus *et al.*, 2006; Kanemaki and Labib, 2006). Thus, the replication initiation and progression complexes are large molecular entities that contain numerous potential targets for CDK and DDK, but most studies have first focused on the MCM complex

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Abbreviations used: DDK, Dbf4-dependent kinase; preRC, prereplicative complex; MCM, minichromosome maintenance; S-CDK, S phase cyclin-dependent kinase.

because it is well conserved among eukaryotes and carries helicase activity.

Several *in vivo* phosphorylation sites in the N-terminal region of Mcm2, 4, and 6 have been mapped, which can be phosphorylated *in vitro* either by CDK or DDK (Komamura-Kohno *et al.*, 2006; Masai *et al.*, 2006; Montagnoli *et al.*, 2006; Sheu and Stillman, 2006). However, mutation of these sites to either Ala or Glu does not cause lethality, and the importance of these modifications for the proper execution of S phase remains to be evaluated. Recently, a role for DDK-dependent Mcm4 phosphorylation in promoting interaction with Cdc45 was demonstrated (Masai *et al.*, 2006; Sheu and Stillman, 2006). There is also evidence suggesting that Mcm4 phosphorylation by CDK might be inhibitory: in *Xenopus*, Mcm4 hyperphosphorylation by CDK was correlated with decrease of its binding to chromatin (Hendrickson *et al.*, 1996; Findeisen *et al.*, 1999) and the *in vitro* helicase activity of Mcm4-6-7 was inhibited when Mcm4 was phosphorylated by Cdk2 (Ishimi *et al.*, 2000). *In vivo* studies have determined that budding yeast Clb5,6-Cdk1 acts positively on DNA replication by phosphorylating Sld2 (Masumoto *et al.*, 2002) and the DNAPolε subunit Dpb2 (Kesti *et al.*, 2004). An Sld2 mutant in which all CDK phosphoacceptor sites are changed to Ala is lethal, shows strong defects in S phase progression, and it was demonstrated that phosphorylation of Thr84 is solely responsible for stabilizing the Sld2-Dpb11 interaction (Tak *et al.*, 2006). A breakthrough came from the recent discovery that phosphomimetic forms of Sld2 combined to constitutive Sld3-Dpb11 complex formation can bypass all minimal requirement of CDK for DNA replication (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). That Sld2 and Sld3 phosphorylation is sufficient implies that phosphorylation of MCM by CDK is not essential for DNA replication. Although not a prime player, the MCM complex is clearly targeted by CDK and DDK in several eukaryotes, where fine-tuning of replisome assembly and helicase activity might be biologically important. In contrast, *Archaea* use only a subset of initiation factors found in eukaryotes and neither orthologues of Sld3, Cdc45, Sld2, and Dpb11 nor of CDK/DDK can be found. The homohexameric MCM complex from *Methanobacterium thermoautotrophicum* has strong helicase activity *in vitro*, whereas MCM complexes or sub-complexes isolated from eukaryotes have, at best, a weak activity (Kelman *et al.*, 1999; Chong *et al.*, 2000; Lee and Hurwitz, 2001; Shin *et al.*, 2003). Interestingly, archaeal Mcm proteins also lack the S/T-rich N-terminal extensions of eukaryotic Mcm2, 4, and 6, which are proposed targets for regulation by CDK and DDK. Here, we provide *in vivo* evidence that phosphorylation of CDK consensus sites within the N-terminus of *S. cerevisiae* Mcm4 contributes to efficient origin firing. We also find that preventing Mcm4 N-ter phosphorylation is severely deleterious when combined to gain of function DDK mutations, suggesting that a proper balance between CDK and DDK activities on the MCM complex is necessary for efficient replisome assembly or progression.

MATERIALS AND METHODS

Plasmids and Yeast Strains

tetCDC7 plasmid (D577) was described previously (Nougarede *et al.*, 2000), and it is a YCplac22(TRP1) derivative. The *DBF4* open reading frame (ORF) was inserted into pCMI89(*URA3*) (Gari *et al.*, 1997) to obtain the *tetDBF4* plasmid. Mutations of putative CDK phosphorylation sites in ScMcm4 (Ser 7, 17, 32, 69, and 145) were generated using the QuikChange mutagenesis kit (GE Healthcare, Chalfont St. Giles, United Kingdom), substituting serines for alanines. Mutation of Ser7 was marked with a *HaeIII* restriction site, and Ser17 by a *KasI* site. Integration of all five mutations at the *MCM4* locus to generate

the *mcm4-5A* allele was done by direct gene replacement. A *URA3* marker was first integrated 350 base pairs before the start codon of the *MCM4* ORF. Transformation with a mutant *MCM4* gene fragment and selection with 5-fluoroorotic acid allowed popping out of the *URA3* gene and integration of the mutated allele (strain E1448). The presence of mutations was verified by DNA sequencing. The *bob1-1* allele was marked by replacing nucleotides 25–128 downstream of *MCM5* stop codon by the *Kluyveromyces lactis* *TRP1* gene. Strains expressing tagged versions of wild-type Mcm4 and mutant Mcm4-5A proteins were constructed by replacing the stop codon of the *MCM4* and *mcm4-5A* genes by the TEV-PrA-7His-*Sp.his5*⁺ cassette of plasmid pYM10 (Knop *et al.*, 1999). A 3HA-tagged *CLB5* gene under the control of the *GALS* promoter was generated by inserting the *GALS*-3HA-*nat* cassette of pYM-N32 (Janke *et al.*, 2004) just downstream of the initiator ATG codon of the *CLB5* gene. Chemical inhibition of CDK activity in the *cdc28-as1* strain was done in YEPD medium containing 0.5 mM 4-amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1-NMPP1) (Bishop *et al.*, 2000). Table 1 lists yeast strains used in this study.

DNA Combing

In vivo 5-bromo-2'-deoxyuridine (BrdU) incorporation, DNA combing and detection were performed as described previously (Lengronne *et al.*, 2001), except that BrdU stretches and DNA fibers were labeled concomitantly with two different fluorophores. BrdU was detected with a rat anti-BrdU antibody (clone BU-75; SeraLab, Crawley, United Kingdom) and a secondary antibody coupled to Alexa 488 (Invitrogen, Paisley, United Kingdom), whereas DNA was revealed with a mouse anti-guanosine antibody (clone GK-21; Argene, Varilhes, France) and a secondary antibody coupled to Alexa 546 (Invitrogen). Interorigin distances (IODs), defined as the distance between the center of two successive colinear BrdU tracks, were measured and plotted as a distribution of IOD range categories. Box-and-whiskers plots were generated using GraphPad Prism software to visualize the main parameters of the distributions. Hypotheses that two distributions are equal or not were verified using the Mann-Whitney statistical test.

PhosTag Western Blot Analysis

Whole-cell extract proteins (15 µg) prepared using the Tri-chloro-acetic acid (TCA) method were loaded on standard 6% SDS-polyacrylamide gel electrophoresis (PAGE) gels (10 × 10 × 0.08 cm) containing 25 µM PhosTag ligand (AAL-107; NARD Institute, Amagasaki, Japan) and 50 µM MnCl₂, according to Kinoshita *et al.* (2006), with special care to avoid any traces of phosphate in buffers or molecular weight markers. Gels were run at 40 mA for 1 h 30 until bromophenol blue runs out, rinsed twice for 10 min in transfer buffer (Tris-glycine, SDS, and ethanol) containing 1 mM EDTA to chelate MnCl₂, and once in the same buffer without EDTA. Proteins were transferred on ProTran membrane (Whatman Schleicher and Schuell, Dassel, Germany) by semidry blotting for 75 min at 0.1 mA/cm². The protein A tag was revealed using peroxidase anti-peroxidase (PAP) antibody (P1291; Sigma Chemical, Poole, Dorset, United Kingdom) at dilution 1:4000.

RESULTS

The N Terminus of Yeast Mcm4 Is Phosphorylated *In Vivo* on CDK Sites

The N-terminal domain of eukaryotic Mcm4 contains a cluster of putative CDK phosphorylation sites that is conserved throughout evolution (Figure 1). This feature is striking in view of the weak overall sequence conservation of this domain. In *S. cerevisiae*, there are two SPxK/R (S7 and S145) and three SP motifs (S17, S32, and S69) within the first 150 residues. Interestingly, all five CDK motifs are preceded by one or two serine residues that, according to Masai *et al.* (2006) and Montagnoli *et al.* (2006), could correspond to DDK phosphoacceptor sites. This juxtaposition of potential DDK/CDK phosphoacceptors is a conserved feature of Mcm4 N termini as all species aligned in Figure 1 contain at least four of them. The remainder of the protein consists mostly of highly conserved domains (AAA⁺ helicase motifs) with very few potential CDK sites. We performed phosphoproteomic analysis with an allele of *MCM4* tagged at its C-terminus with protein A and introduced at the natural locus. However, assessing the *in vivo* phosphorylation status of yeast Mcm4 turned out to be difficult because the protein did not show obvious mobility shift during the cell cycle, in standard or modified western blot conditions (Figure 2B, top). Two-dimensional gel electrophoresis and mass

Table 1. List of strains used in this study

Strain	Relevant genotype	Origin
E001	<i>MATa, ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	W303-1A
E718	<i>MATa, bob1-1</i>	C. Hardy (Vanderbilt University)
E1000	<i>MATa, ura3::URA3/GPD-TK(7×)</i>	Lengronne <i>et al.</i> (2001)
E1448	<i>MATa, mcm4-5A</i>	This study
E1823	<i>MATa, mcm4-5A, bob1-1, ura3::URA3/GPD-TK(7×)</i>	This study
E1825	<i>MATa, bob1-1, ura3::URA3/GPD-TK(7×)</i>	This study
E1826	<i>MATa, mcm4-5A, ura3::URA3/GPD-TK(7×)</i>	This study
E1968	<i>MATa, clb5::HIS3, clb6::LEU2</i>	This study
E1971	<i>MATa, clb5::URA3, clb6::LEU2</i>	This study
E2476	<i>MATa, clb5::URA3, clb6::LEU2, bob1-1::TRP1</i>	This study
E2486	<i>MATa, mcm4-5A, bob1-1</i>	This study
E2605	<i>MATa, clb5::HIS3, clb6::LEU2, SWI5::SWI5pr-CLB5::URA3</i>	This study
E2606	<i>MATa, clb5::HIS3, clb6::LEU2, bob1-1::TRP1, SWI5::SWI5pr-CLB5::URA3</i>	This study
E2726	<i>MATa, MCM4::TEV-PrA-7His/Sphis5⁺</i>	This study
E2729	<i>MATα, clb6::LEU2, bob1-1::TRP1, swe1::KanMX, clb5::CLB3</i>	This study
E2731	<i>MATα, clb5::URA3, clb6::LEU2, bob1-1::TRP1, swe1::KanMX</i>	This study
E2738	<i>MATa, mcm4-5A::TEV-PrA-7His/Sphis5⁺</i>	This study
E2756	<i>MATα, clb6::LEU2, bob1-1::TRP1, swe1::URA3, clb5::CLB4</i>	This study
E2781	<i>MATα, clb6::LEU2, bob1-1::TRP1(KI), swe1::HIS5, clb5::CLB2</i>	This study
E2799	<i>MATa, clb6::LEU2, bob1-1::TRP1, swe1::KanMX</i>	This study
E3200	<i>MATα, MCM4::TEV-PrA-7His/Sp.his5⁺, GAL5-3HA-CLB5/nat, clb6::LEU2</i>	This study
E3215	<i>MATα, mcm4-5A::TEV-PrA-7His/Sp.his5⁺, GAL5-3HA-CLB5/nat, clb6::LEU2</i>	This study
E3218	<i>MATa, MCM4::TEV-PrA-7His/Sphis5⁺, cdc28-as1</i>	K. Shokat (University of California, San Francisco); this study

All strains are congenic or at least backcrossed four times to W303-1a.

spectrometry analyses were also unsuccessful (data not shown), perhaps due to the fact that only a small fraction of cellular MCM molecules is phosphorylated in vivo (Sheu and Stillman, 2006). Recently, a phosphate ligand (PhosTag) was introduced, which significantly slows down the migration of phosphoproteins in SDS-PAGE (Kinoshita *et al.*, 2006). Using PhosTag, we found very reproducibly that ~10–20% of Mcm4 molecules migrate more slowly, depending on cell cycle position (Figure 2B, bottom). These Mcm4-specific (Figure 2A) slower migrating species were present

in α -factor-arrested cells, decreased at 15 min and reached their maximum 30 min after release, concomitant with DNA replication. Better inspection of samples run without PhosTag (Figure 2B, top) reveals a broadening of the Mcm4 band at 30min, which likely corresponds to the phosphorylated forms seen using PhosTag. These forms decreased in G2/M to increase again upon S phase in the following cycle (75 min). We conclude that a fraction of yeast Mcm4 is phosphorylated in vivo during S phase. To test whether this phosphorylation depends on CDK sites within the N ter-

Sc	MSQQSSPTKEDNNSSSFVVPNPDSVPPQLSSP	54
Sp	MSSSQSGRANELRTPGRANSSSREAVDSPLFFPAS	52
Ca	MSPSPQSTNQNNNPADSLERESGSIQSQQPPIVSSPLFFNSNPGSDIGNFSQSQS	60
Hs	MSPASTPSRRGSRRRGRATPAQTPRSEDARSSPSQRRRGEDSTSTGELQP	50
Mm	MSPASTPSRRGSRRRGRVTPTQSLRSEESRSSPNRRRRGED.SSTGELLP	49
Xl	MSPSTPSRRRVNVEAVTLLQLLVERKCSLLRHRKDGQKIPHLVFSFCP	50
Dm	MSPARSPSVGGATPKQGARTPTRGIASQDVETPMRMGPGRAVRPSDNIS	50
At	MASDSSLGNTNDGPPSPGENVSSPIENTYSSFAALHRRRRGRSSPTPTQFA	50
Sc	LSQGEGRNIRAAIGSSPLNFPSSSRQNSDVFQSQGRQGRIRSSASASGRSRYHSDLRSDR	114
Sp	TPLASPLLFESSSPGNIPQSSRSHLLSQRNDLFLDSSSRTPRSTRRGDIHSSVQMS	112
Ca	QSQSQ...RRNDISSPLHYTSSAQPTSDIGGFDSQRSARVQDVGRIMRAQRSDVTDVS	117
Hs	MPTSPGVLDQSTAAQDVLFSPPQMHSALPLDFDVSSPLTYGTPSSR.VEGTPRSGVRG	109
Mm	MPTSPGADLQSPPAQNALFSPPQMHSALPLDFDVSSPLTYGTPSSR.VEGTPRSGVRG	108
Xl	CQPHLQETFRVP.....CFLALAPSRHSAHQSELDLSSPLTYGTPSSR.VEGTPRSGVRG	104
Dm	LPPTSPGNISLP.....ATSPARGLGANMSEIDLSSPLNYGTPSSMGSIPTPRSGIRG	103
At	TPPPPSRLASS.....NSTPPTSRSARSKGRNGHGGGGGGGGDPGTPMSTDEP	103
Sc	ALPTSSSSSLGRNGQNRVHMRRNDIHTSDLSPPRIIVDFDTRSGVNTLDTSSSSAPPSEAS	174
Sp	PSRRREVDPQRPGVSTFSSLLFS.....GSDALTFQAHPSSEVAD	153
Ca	SPQRSSRYFTQGRNGGPSNLN.....SSTSAQFSTDPAPEND	154
Hs	TPVVRQRPDLGSAQKGLQVDLQS.....DGAAEDIVASEQSL	146
Mm	TPVVRQRPDLGSAQKGLQVDLQS.....DGAAEDIVPSEQSL	145
Xl	TPARQRADLGSAQKGLQVDLHS.....DQPAEELVTSEQSL	141
Dm	TPLRARPDITDKRIRQVAIGGG.....SGLEPIPEKGSSETDPVSESSQ	148
At	LP.....SSDDGEEDGGDDTPTTFV	123

Figure 1. Multiple alignment of the N-terminal domain of Mcm4 from different species. Sc, *S. cerevisiae*; Sp, *S. pombe*; Ca, *Candida albicans*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*. Potential CDK phosphorylation sites are marked by black boxes.

Figure 2. The N-terminal extension of Mcm4 is phosphorylated *in vivo* during S phase in a CDK-dependent manner. (A) Specificity of Western blot detection of Mcm4 tagged with protein A. Whole-cell extracts from untagged strain (lane 1), three MCM4-PrA transformants (lanes 2–4), and an unrelated strain expressing SmB1-TAP (lane 5) detected on Western blots by using PAP antibody and enhanced chemiluminescence. All signal derives from the PrA-tagged protein. (B–E) Strains of the indicated genotype expressing a single copy of either MCM4 or *mcm4-5A* tagged with protein A were arrested with α -factor and released in YPD medium at 30°C (B and C) or in YPD containing 0.5 mM 1-NMPP1 (D and E). Samples were taken at the indicated times and analyzed for DNA content by flow cytometry (left) and for Mcm4 phosphorylation using SDS-PAGE run with or without 25 μ M PhosTag as indicated. The vertical bar and asterisk indicate slower migrating phospho-Mcm4 species on Western blots.

minus, serines within all five SP or SPxK/R motifs (S7, 17, 32, 69, and 145) were substituted to alanine. This allele (*mcm4-5A*) was subjected to PhosTag analysis as mentioned above. Mutation of these sites caused disappearance of all slower migrating bands (Figure 2C), demonstrating that Mcm4 phosphorylation *in vivo* depends on one or more of these five CDK sites clustered within Mcm4's N-terminus. However, the complex pattern of Mcm4 phosphorylation during the cell cycle precluded any simple assessment of the kinase phosphorylating these sites. Specific inhibition of CDK during an α -factor release by using a small molecule (1-NMPP1) in a *cdc28-as1* strain led to a complete disappearance of slower migrating bands during the first 30 min of the time course (Figure 2E). Although these cells never exited G1 (no budding, no DNA replication), slower migrating bands reappeared at later times, indicating that Mcm4 phosphorylation can also occur in a CDK-independent manner. This suggests that several kinases can phosphorylate Mcm4 but that CDK inhibition prevents or slows down the initial phosphorylation of Mcm4 in late G1.

mcm4-5A Is Synthetic Lethal with clb5,6 Δ and mcm5/bob1

The biological importance of these five CDK phosphoacceptor sites (S7, S17, S32, S69, and S145) within Mcm4 was

assessed using flow cytometry, cell viability, and minichromosome maintenance assays, which did not point toward obvious DNA replication defects in the *mcm4-5A* mutant strain (data not shown). Thus, these sites cannot be essential CDK targets, but it does not rule out that Mcm4 sites may act synergistically with other targets. Indeed, we found that the *mcm4-5A* allele is lethal in a strain lacking the S phase cyclins Clb5 and 6, which on its own has normal viability despite delayed DNA replication (Figure 3A) (Schwob and Nasmyth, 1993). Using a conditional allele of *CLB5* driven by the *GALs* promoter, we were able to test the consequences for DNA replication and cell division of depleting Clb5 in a *clb6 mcm4-5A* strain. Figure 3B shows that *GALs-CLB5 clb6 mcm4-5A* cells on glucose replicate DNA more slowly than *GALs-CLB5 clb6* control cells (compare the 45- and 150-min times in both strains). The triple mutant cells underwent one or two additional divisions and then died without ever forming a colony (Figure 3A). The additive effects (lethality) of *mcm4-5A* and *clb5,6 Δ* mutations implies both that the N-terminal Mcm4 sites can be phosphorylated by other kinases, possibly M phase CDKs and that Clb5,6-CDK (obviously) targets other key proteins. The lethality of *mcm4-5A clb5,6 Δ* cells would thus stem from the impossibility to phos-

Figure 3. Synthetic lethality of *mcm4-5A* and *clb5,6Δ*. (A) Serial fivefold dilutions of strains E3013 (*GALs-CLB5 clb6Δ*), E3192 (*mcm4-5A*), E3205 (*GALs-CLB5 clb6Δ mcm4-5A*), and E3208 (*GALs-CLB5 clb6Δ mcm4-5A bob1-1*) were spotted on YGal and YD (Glu) plates and grown for 2–3 d at 30°C. (B) Strains E3200 (*GALs-CLB5 clb6Δ*) and E3215 (*GALs-CLB5 clb6Δ mcm4-5A*) were grown in YEPGal medium, arrested in G1 with α -factor, and depleted for Clb5 by addition of 2% Glu to the medium 30 min before release from G1 arrest. Samples were taken at the indicated times and analyzed for S phase progression using flow cytometry.

phorylate Mcm4 combined to the absence (or delay) of phosphorylation of replication factors normally targeted by Clb5,6-Cdk1, such as for example Sld3 (Zegerman and Difley, 2007).

We also combined the *mcm4-5A* allele with various other mutations in replication initiation factors (including putative CDK sites within Mcm2 and Mcm10). One double mutant, *mcm4-5A bob1*, turned out to be cold-sensitive (cs) for growth (Figure 4A). *bob1-1* is an allele of MCM5 (changing Pro83 to Leu) that bypasses the requirement of *CDC7* and *DBF4* for DNA replication (Hardy *et al.*, 1997). The *mcm4-5A bob1* double mutant ceases cell proliferation at 22°C (and below) after one to three cell divisions. To see whether these cells have problems to initiate DNA replication, *bob1* and *mcm4-5A bob1* cells were synchronized with α -factor at permissive temperature (32°C) to allow preRC formation, and then they were released at 22°C (restrictive temperature). Flow cytometry analysis revealed that although S phase started on schedule (30 min after α F-release) in the double mutant, it progressed very slowly, reaching apparent completion only at 105 min (instead of 60 min in the *bob1* control; Figure 4B). Figure 4C shows that the *mcm4-5A bob1* double mutant is also highly sensitive to hydroxyurea (HU) at permissive temperature. These cells did not die in the first cell cycle (unlike *rad53* mutants on HU) but formed microcolonies composed of 10–20 cells, suggesting that they are capable of exiting mitosis under chronic HU exposure but suffer from gradual loss of viability.

Figure 4. Phenotypes of the *mcm4-5A bob1-1* mutant. (A) Serial fivefold dilutions of strains E001 (WT), E1448 (*mcm4-5A*), E718 (*bob1-1*) and E2486 (*mcm4-5A bob1-1*) were spotted on YPD plates and grown at 32 or 22°C. (B) *bob1-1* and *mcm4-5A bob1-1* strains grown in rich medium were arrested in G1 with α -factor at 32°C and released at 22°C by pronase addition. DNA content was analyzed by flow cytometry. BI, budding index. (C) Serial fivefold dilutions of WT (E001), *mcm4-5A* (E1448), *bob1-1* (E718) and *mcm4-5A bob1-1* (E2486) strains were spotted on YPD plates containing or not 66 mM HU and grown at 31°C. (D) MCM4 (wild type and E001) and *mcm4-5A* (E1448) strains containing plasmids pCM189(*URA3*) (*tet-U*) and YCplac22(*TRP1*) (*YCp-W*), containing or not the *DBF4* or *CDC7* genes under control of a tetracycline-repressible promoter were spotted as serial fivefold dilutions on synthetic medium lacking uracil and tryptophan and containing (transcription OFF) or not (ON) 2 μ g/ml doxycycline at 22°C.

mcm4-5A bob1 Double Mutants Have Replication Initiation Defects

The slow S phase in the *mcm4-5A bob1* mutant at 22°C could be due either to a decrease of the number of active origins or to elongation proceeding at a slower pace. To monitor origin firing efficiency, we analyzed BrdU-labeled DNA fibers straightened on a microscope glass slide by using DNA combing (Lengronne *et al.*, 2001). On release from α -factor at 22°C into medium containing HU, BrdU gets incorporated into short 10- to 20-kb regions surrounding origins (Figure 5A). The density of active origins was then calculated by measuring the distance separating the center of two successive BrdU tracks (IOD) in >200 fibers. Figure 5B shows that the mean IOD in the *mcm4-5A bob1* double mutant is more than twice that of *bob1* and wild-type strains (90 kb instead of 42 kb), implying that the number of fired origins (at least for the subset of early origins) is significantly lower in the double mutant. The notion that

C

Strains compared	median ₁ ≠ median ₂ ? (p < 0.05)
WT vs <i>bob1-1</i>	No
WT vs <i>mcm4-5A</i>	Yes
WT vs <i>bob1-1, mcm4-5A</i>	Yes
<i>mcm4-5A</i> vs <i>bob1-1, mcm4-5A</i>	Yes

Figure 5. DNA combing analysis of origin firing. (A) WT, *bob1-1*, *mcm4-5A*, and *bob1-1 mcm4-5A* strains containing seven copies of the thymidine kinase gene (E1000, E1826, E1825, and E1823, respectively) were grown in rich medium, arrested with α -factor at 32°C, and released for 90 min at 22°C in medium containing 200 μ g/ml BrdU and 0.2 M HU. Genomic DNA was extracted from cells and DNA fibers combed on silanized glass slides. DNA fibers (red signals) and BrdU-substituted regions surrounding origins activated in early S phase (green signals) were visualized by fluorescence microscopy by using anti-guanosine (Argene) and anti-BrdU (BU-75; SerLab) antibodies, respectively. (B) Distribution of IODs, plotted as histograms. (C) The IOD distributions were compared between each strain using a Mann–Whitney statistical test, which determines whether the medians of the distributions are significantly different.

mcm4-5A bob1 cells have a slow S phase because of initiation, not elongation defects is supported by the absence of a significant delay in S phase completion when *mcm4-5A bob1* cells are first arrested in HU at permissive temperature (allowing for

early origins to fire) and then released at restrictive temperature (Supplemental Figure S1). We conclude that the lengthening of S phase in the *mcm4-5A bob1* double mutant is almost entirely due to a failure to activate origins.

mcm4-5A bob1 Replication Initiation Defects Stem from DDK Gain of Function

One trivial explanation for the synthetic effects between *mcm4-5A* and *mcm5/bob1* could be that each mutation causes a slight loss of function that together would affect the functionality of the MCM complex. A more attractive hypothesis is that the N-termini of Mcm4 and Mcm5 (where the *mcm4-5A* and *bob1* mutations reside) integrate regulatory signals conveyed by CDK and DDK. In this scenario, contradictory inputs brought about by the *mcm4-5A* (loss of CDK function) and *bob1* (DDK bypass) mutations would make the initiation mechanism inefficient. In keeping with the latter hypothesis (contradictory inputs) for the lethality of *mcm4-5A* and *bob1*, we found that ectopic DDK expression recapitulates the phenotypes obtained with *mcm5/bob1*. Plasmids bearing the *CDC7* and *DBF4* genes under control of a Tet-repressible promoter were introduced in the *mcm4-5A* strain. As shown in Figure 4D, the *mcm4-5A* mutant did not grow at 22°C in the absence of doxycycline, when both *CDC7* and *DBF4* are coexpressed. This lethality was not observed when only one of the two DDK subunits was overexpressed, or at 32°C at which temperature cells grew equally well in the presence or absence of doxycycline (data not shown). These findings indicate that the lethality of the *mcm4-5A bob1* strain likely stems from precocious or constitutive DDK-

dependent activation of the MCM complex, not from a loss of function of the Mcm5^{Bob1} subunit. The cold-sensitive phenotype suggests that, in *mcm4-5A bob1* cells at 22°C, a fraction of preRCs might be locked in a conformation that is refractory to inducers of DNA replication.

CDK and DDK Actions Need to Be Coordinated for Efficient Origin Activation

These observations prompted us to evaluate the possibility that the *mcm4-5A bob1* lethality was due to an imbalance between CDK and DDK activities. One possibility is that forced activation of the preRC by DDK without previous Mcm4 phosphorylation by CDK leaves preinitiation complexes in a state that is unstable or refractory to origin firing. If this scenario were correct, delaying the activation of S phase CDK in a *bob1* background should produce the same phenotype as the *mcm4-5A bob1* double mutant. Deletion of *CLB5* and *CLB6* leads to a 30- to 40-min delay in the initiation of DNA replication, which is then triggered by Clb1–4 cyclins (Schwob and Nasmyth, 1993). As predicted by the above-mentioned hypothesis, the *clb5,6Δ bob1* triple mutant was also found cold sensitive for growth (Figure 6A), with DNA replication progressing very slowly at the nonpermissive temperature (Figure 6B). Moreover, the *clb5,6Δ bob1* triple mutant showed HU sensitivity similar to that of the

Figure 6. *clb5,6Δ bob1-1* cells show defects very similar to those of *mcm4-5A bob1-1*. (A) Serial fivefold dilutions of strains E001 (WT), E718 (*bob1-1*), E1971 (*clb5,6Δ*), and E2476 (*clb5,6Δ bob1-1*) were spotted on YPD plates and grown at 32 or 22°C. (B) *bob1-1* and *clb5,6Δ bob1-1* strains grown in rich medium were arrested in G1 with α -factor at 32°C and released at 18°C by pronase addition. DNA content was analyzed by flow cytometry. BI, budding index. (C) Serial fivefold dilutions of *clb5,6Δ* and *clb5,6Δ bob1-1* strains were spotted on YPD plates containing or not 0.2 M HU and grown at 31°C. (D) E001 (WT) and E1448 (*mcm4-5A*) strains containing plasmids *tet(U)* (URA3), YCp(W) (TRP1), containing or not the *DBF4* or *CDC7* genes under control of a tetracycline-repressible promoter were spotted as serial fivefold dilutions on synthetic medium lacking uracil and tryptophan and containing (transcription OFF) or not (ON) 2 μ g/ml doxycycline at 22°C.

Figure 7. SWI5pr-CLB5 but not *clb5::CLB2*, 3, or 4 suppresses cold lethality of *clb5,6Δ bob1-1*. Serial fivefold dilutions of strains E1971 (*clb5,6Δ*), E2476 (*clb5,6Δ bob1-1*), E2605 (*clb5,6Δ SWI5pr-CLB5*), and E2606 (*clb5,6Δ SWI5pr-CLB5 bob1-1*) (A), or *clb6Δ swe1Δ bob1-1* strains containing either wild-type *CLB5* (E2799), *clb5Δ* (E2731) or *CLB2*, *CLB3*, or *CLB4* under control of the *CLB5* promoter (E2781, E2729, and E2756, respectively) (B) were spotted on YPD plates and grown at 31 or 18°C.

original *mcm4-5A bob1* mutant (Figure 6C), indicating that ablation of Clb5 and 6 recapitulates the effects caused by removing Mcm4 phosphoacceptor residues in a *bob1* context. We further explored whether, in this situation of delayed CDK activation, hyperactivation of DDK could substitute for the *bob1* mutation. Indeed, Figure 6D shows that *clb5,6Δ* cells cannot grow when both *CDC7* and *DBF4* are overexpressed. The strong similarity of phenotypes obtained with *mcm4-5A* and *clb5,6Δ* in conditions of DDK bypass or up-mutations is consistent with residues S7, S17, S32, S69, and S145 of Mcm4 being *in vivo* targets of CDK. It also suggests that S-CDK and DDK must act in a balanced and coordinated manner to fire origins in an efficient way.

Mitotic Cyclins Cannot Substitute for Clb5,6 in *bob1* Cells

In the experiments described above (Figure 6), the lethality of *bob1 clb5,6Δ* cells at low temperature could stem either from a delay in phosphorylating S-CDK substrates (if mitotic cyclins can substitute) or a complete lack thereof (if Clb1–4/Cdk1 cannot productively phosphorylate these targets). To distinguish between these possibilities, we introduced in the *bob1 clb5,6Δ* strain a version of *CLB5* under control of the G2/M-specific *SWI5* promoter, which causes Clb5-Cdk1 kinase activity to occur at the same time as that of Clb2-Cdk1 (Supplemental Figure S2). Figure 7A shows that the *SWI5pr-CLB5* allele completely suppressed the cold lethality of the *bob1 clb5,6Δ* mutant. Thus, although Clb5 expressed in G2/M can rescue the replication defects of the *bob1 clb5,6Δ* triple mutant, mitotic Clb1–4 cyclins that are expressed and active at the same time cannot. We conclude that it is not the timing of CDK activation that is critical for the viability of *bob1* cells, rather the specificity of the cyclin–Cdk complex. Clearly, Clb5,6/Cdk1 does something to *bob1* cells that Clb1–4/Cdk1 cannot. To strengthen this conclusion, we performed the reciprocal experiment by expressing mitotic cyclins at a time matching that of the Clb5,6 S phase

Figure 8. S phase is slower in the *mcm4-5A* mutant. E001 (WT) and E1448 (*mcm4-5A*) strains grown in rich medium were arrested in G1 with α -factor at 32°C and released at 18°C by pronase addition. DNA content was analyzed by flow cytometry. BI, budding index.

cyclins. To this aim, *CLB2*, 3, and 4 open reading frames were each introduced at the *CLB5* locus (under control of the *CLB5* promoter) in a *bob1 clb6Δ swe1Δ* strain. It was shown previously that *SWE1* deletion significantly increases the ability of *clb5::CLB2*, 3, or 4 to drive S phase to almost wild-type kinetics in a *clb5,6Δ* strain (Hu and Aparicio, 2005). Furthermore, it is known that Clb2 protein made from the *CLB5* promoter is expressed at similar levels and timing as endogenous Clb5, while carrying stronger kinase activity (Cross *et al.*, 1999; Loog and Morgan, 2005). Despite this, we found that none of the mitotic cyclins expressed early could suppress the cold sensitivity of the *bob1 clb5,6Δ swe1Δ* strain (Figure 7B). Thus, although either S or M phase cyclins can trigger DNA replication in wild-type cells, only Clb5 and 6 can do so in a strain where DDK regulation has been bypassed, suggesting that Clb5,6/Cdk1 has the unique property to interface with DDK activity.

Phosphorylation of the Mcm4 N Terminus Improves Origin Firing Efficiency

What could be the role of Mcm4 N-ter phosphorylation during the normal process of initiation? Although the *mcm4-5A* mutation did not confer obvious growth defects in otherwise wild-type cells, careful analysis of S-phase progression by flow cytometry and DNA combing revealed a significant and reproducible lengthening of S phase in the *mcm4-5A* mutant strain at 18°C. DNA replication was completed only 120 min after release from G1 compared with 90 min in wild type (Figure 8). This lengthening was also present at 22°C, but less important (data not shown). Using DNA combing, we found that the mean distance between active origins was 61 kb in the *mcm4-5A* single mutant at 22°C, compared with 42 kb in wild-type and *bob1* cells (Figure 5 and Supplemental Figure S3), indicating that chromosomes are duplicated from fewer origins in *mcm4-5A* cells. These results demonstrate that, although not essential in laboratory conditions, the phosphorylation of the Mcm4 N-terminus contributes to the efficacy of origin firing.

DISCUSSION

Mcm4 N-Ter Phosphorylation and the Initiation of DNA Replication

Mcm4 is highly conserved in eukaryotes. In multiple alignments covering divergent species, the similarity is high throughout most of its length, reaching 61% identity over a

137-amino acids stretch. The similarity drops dramatically, however, within the first 170 residues of Mcm4, which are rich in potential CDK phosphorylation sites. Using phosphospecific antibodies Ishimi and coworkers showed that at least seven of the 11 N-terminal S/TP sites are phosphorylated in vivo in human cells (Komamura-Kohno *et al.*, 2006). In vitro studies revealed that these phosphorylations are CDK-dependent (Ishimi and Komamura-Kohno, 2001). Finally, phosphopeptide mapping showed that CycB-Cdk1 was responsible for most if not all Mcm4 phosphorylation in M phase *Xenopus* extracts (Hendrickson *et al.*, 1996; Pereverzeva *et al.*, 2000). Here, we show that a fraction of Mcm4 molecules is phosphorylated during S phase in *S. cerevisiae* (Figure 2). Although we could not provide a formal demonstration that CDK is indeed responsible for these phosphorylations, this idea is supported by the strong similarities between the phenotypes caused by the elimination of five CDK phosphoacceptor residues within the N-terminus of ScMcm4 and those obtained by deleting the S-phase cyclins, when these mutants are combined to DDK gain-of-function mutations.

We provide the first in vivo evidence that phosphorylation of Mcm4 contributes to an efficient initiation of DNA replication. This phosphorylation of Mcm4 is not essential for initiation because the *mcm4-5A* mutant strain, which lacks all five CDK phosphoacceptor sites, displays only subtle replication defects in laboratory conditions. Yet, at temperatures of $\leq 22^{\circ}\text{C}$, which are common for yeast ecotypes, the *mcm4-5A* allele causes a clear lengthening of S phase, which correlates with a reduced number of fired origins determined by DNA combing. Interestingly for our understanding of the mechanism of replication initiation, we show that more profound effects are observed when the *mcm4-5A* allele is combined to mutations that bypass (*mcm5/bob1*) or up-regulate (ectopic Cdc7-Dbf4 expression) DDK activity. In this context, *mcm4-5A* cells proceed exceedingly slowly through S phase at low temperatures, leading to cell death within the first three cell divisions. The terminal morphology consists of large dumbbell cells with DNA trapped in the neck or stretched between the mother and daughter cells (data not shown). The decreased origin firing measured on single DNA molecules in the *mcm4-5A bob1* strain points to an activating, not inhibitory, role of Mcm4 phosphorylation by CDK in initiating DNA replication. BrdU tracks were not shorter, nor was S phase significantly lengthened when *mcm4-5A bob1* cells were shifted to restrictive temperature after initiation, arguing against a role of Mcm4 N-ter phosphorylation in the elongation of DNA synthesis. In contrast to Mcm4 that we show here to only contribute to efficient origin firing, Sld2 and Sld3 are essential to promote DNA replication in a CDK-dependent manner (Tak *et al.*, 2006; Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). We suggest that, in addition to promoting the formation of Sld2-Dpb11 and Sld3-Cdc45 complexes required for recruitment of GINS and replisome progression complexes, S-CDKs also perform a nonessential regulatory function on the MCM complex that interfaces with DDK function.

Our finding that Clb5 expressed late in the cell cycle (from the *SWI5* promoter), but not any of the mitotic cyclins Clb2, 3, or 4 expressed early (from the *CLB5* promoter in a *swe1Δ* background), can suppress the cold lethality of the *bob1 clb5,6Δ* mutant indicates that the defects of this strain stem from a lack, not simply a delay of phosphorylation of one or more Clb5,6-specific substrates. It is known that several CDK substrates, among which are some key replication factors such as Orc6, Cdc6, Mcm3, and Sld2, are more efficiently phosphorylated in vitro by Clb5-Cdk1 than by Clb2-

Cdk1 (Loog and Morgan, 2005). This specificity was shown to depend on the presence of a hydrophobic patch in Clb5, and, in some cases, of a Cy or RXL motif in the substrate (Wilmes *et al.*, 2004). Given the genetic evidence presented here, we propose that Mcm4 could be another member of this class of preferential Clb5-CDK targets. However, Mcm4 is not phosphorylated exclusively by S-CDKs, as revealed by the synthetic lethal interaction between the *mcm4-5A* and *clb5,6Δ* mutations and by the residual phosphorylation after chemical inhibition of Cdc28. The kinases responsible for these phosphorylations are likely mitotic CDKs and DDK, which could modify Mcm4 but to a lower level than in the presence of S-CDK. These activities would be sufficient for viability of *clb5,6Δ* cells, but not for the same cells in the context of DDK gain-of-function mutations.

Efficient Origin Firing Entails Coordinated CDK and DDK Action

It is thought that *bob1*, an allele of *MCM5*, bypasses the requirement of *CDC7* and *DBF4* for DNA replication by inducing a conformational change in the MCM complex that mimics activation by Dbf4-Cdc7 kinase. In keeping with this interpretation, it was shown that *bob1* cells exhibit abnormally high amounts of chromatin-bound Cdc45 in α -factor arrested G1 cells (Sclafani *et al.*, 2002). Cdc45 binding to chromatin depends on DDK, and it is therefore very low in wild-type G1 cells. The notion that *mcm4-5A bob1* defects are linked to bypass of DDK regulation is corroborated by our finding that ectopic expression of *CDC7* and *DBF4* in the *mcm4-5A* mutant has the same effect as the *bob1* mutation. Thus, it seems that MCM complexes preactivated by DDK cannot stably promote origin firing, unless Mcm4 is also phosphorylated on its N terminus by S-CDK. This suggests that Mcm4 phosphorylation by CDK compensates for a conformational defect caused by the *bob1* mutation or by DDK hyperactivation (Fletcher and Chen, 2006). Another way to think about these data is that there might be an order in the molecular events leading to preRC activation by CDK and DDK. In this view, forced preRC activation by DDK (either through *bob1* or ectopic DDK expression) without previous Mcm4 phosphorylation by S-CDK might render origins refractory to firing by affecting replisome assembly or stabilization.

Intricacy of CDK and DDK Actions May Provide for Directionality in Replisome Assembly

Why should ectopic DDK activity in conditions in which Mcm4 cannot be phosphorylated by CDK be deleterious for preRC activation? One hint could be that Cdc45 binds to chromatin much earlier in G1 in *bob1* than in wild-type (WT) cells (Sclafani *et al.*, 2002). Binding of Cdc45 before Mcm4 phosphorylation by Clb5,6-Cdk1 might lead to the assembly of an abnormal preinitiation complex that might be locked, at least at low temperatures, in a conformation that cannot easily trigger initiation. Another hypothesis is suggested from recent studies on Mcm2 phosphorylation (Montagnoli *et al.*, 2006), in which six in vivo phosphorylation sites on human Mcm2 have been identified, three dependent on DDK and three on CDK. Two of these modifications affect adjacent serines, with DDK phosphorylating S40 and CDK phosphorylating S41. Strikingly, it was found that although both kinases could phosphorylate their cognate serines (S40 and S41) in vitro when the neighboring Ser was unphosphorylated, only DDK could do so when the other serine was already phosphorylated. That is, CDK is unable to phosphorylate S41 when S40 is already modified. Reciprocally, it was shown that Mcm2 phosphorylation by DDK is

facilitated by prior phosphorylation by CDK (Masai *et al.*, 2000). This suggests that CDK and DDK must act sequentially (CDK first) to phosphorylate hMcm2 on Ser41 and 40, respectively, which also fits the notion that DDK targets S/T residues that are either embedded in acidic stretches or next to residues already carrying (negatively charged) phosphate moieties. Priming of a DDK substrate by prior CDK phosphorylation has been recently demonstrated for the yeast meiotic recombination protein Mer2, and it could be a common mechanism for CDK-DDK coregulation (Wan *et al.*, 2008). Mcm2 has an evolutionary divergent N-terminal domain that contains several such potential DDK-CDK biphenylation SSP (or STP) motifs. In fact, most of the potential CDK sites located in the Mcm4 N-terminal domains of many evolutionary distant species, as well as all five sites that we mutated in ScMcm4 belong to this category (Figure 1). It was shown recently that Mcm4 is phosphorylated by DDK in *Xenopus* and budding yeast (Takahashi and Walter, 2005; Masai *et al.*, 2006; Sheu and Stillman, 2006). Together with our data, these results suggest that one or more subunits of the MCM complex are phosphorylated both by CDK and DDK, whereby CDK might favor the action of DDK but, conversely, in which precocious phosphorylation by DDK might forestall the action of CDK on the preRC. Such a dependency in kinase function might provide for directionality along the sophisticated path of origin firing, which entails the orderly addition of various subcomplexes to the preRC before DNA synthesis actually begins. We propose that the amino-terminal extensions of eukaryotic Mcm2, 4, and 6 integrate regulatory signals conveyed by S-CDK and DDK. Failure to coordinate CDK and DDK activities on the MCM complex might destabilize the replisome and cause abortive firing of replication origins. We speculate that the regulation of the N-terminal extensions of MCM by S-CDK and DDK may also account for the modulation of fork progression rates seen in eukaryotes (Raghuraman *et al.*, 2001) compared with Archaea (Lundgren *et al.*, 2004).

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